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(71) Applicants: THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ARKANSAS [US/US]; 2404 North University Avenue, Little Rock, AR 72207–3608 (US). MOUNT SINAI SCHOOL OF MEDICINE OF NEW YORK UNIVERSITY [US/US]; One Gustave L. Levy Place, New York, NY 10029–6574 (US).

(72) Inventors: BANNON, Gary, A.; 714 St. Michael Place, Little Rock, AR 72211 (US). BURKS, A., Wesley, Jr.; 2400 North Pierce, Little Rock, AR 72207 (US). SAMPSON, Hugh, A.; 19 Carleon Avenue, Larchmont, NY 10538 (US). SOSIN, Howard, B.; 640 Sasco Hill Road, Fairfield, CT 06430 (US). KING, Nina, E.; 7127 Oak Post Lane, #100, Cincinnati, OH 45241 (US). MALEKI, Soheila, J.; 612 Parkway Place, Little Rock, AR 72211 (US). CONNAUGHTON, Cathie; 505 West Ridge Drive, Conway, AR 72032 (US). KOPPER, Randall, A.; 2 Brier Springs, Conway, AR 72032 (US). RABJOHN, Patrick, A.; 11524 Hickory Hill Road, Little Rock, AR 72211 (US). SHIN, David, S.; 2825 Clairmont Court, San Diego, CA 92117 (US). COMPADRE, Cesar, M.; 7215 Gable Street, Little Rock, AR 72205 (US).

(74) Agents: PABST, Patrea, L. et al.; Arnall Golden & Gregory, LLP, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309–3450 (US).

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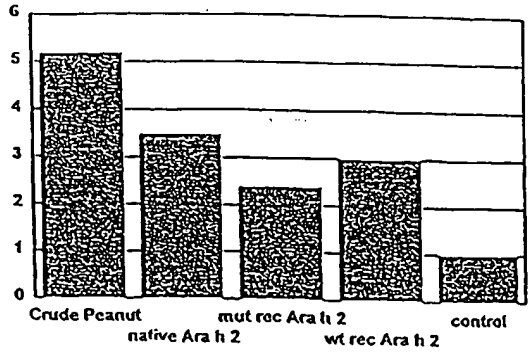


(57) Abstract

It has been determined that allergens, which are characterized by both humoral (IgE) and cellular (T cell) binding sites, can be modified to be less allergenic by modifying the IgE binding sites. The IgE binding sites can be converted to non–IgE binding sites by masking the site with a compound that prevents IgE binding or by altering as little as a single amino acid within the protein, most typically a hydrophobic residue towards the center of the IgE binding epitope, to eliminate IgE binding. The method allows the protein to be altered as minimally as possible, other than within the IgE binding sites, while retaining the ability of the protein to activate T cells, and, in some embodiments by not significantly altering or decreasing IgG binding capacity. The examples use peanut allergens to demonstrate alteration of IgE binding sites. The critical amino acids within each of the IgE binding epitopes of the peanut protein that are important to immunoglobulin binding have been determined. Substitution of even a single amino acid within each of the epitopes led to loss of IgE binding. Although the epitopes shared no common amino acid sequence motif, the hydrophobic residues located in the center of the epitope appeared to be most critical to IgE binding.

METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY

Background of the Invention

The United States government has rights in this invention by virtue of grants from the National Institute of Health RO1-AI33596.

Allergic disease is a common health problem affecting humans and companion animals (mainly dogs and cats) alike. Allergies exist to foods, molds, grasses, trees, insects, pets, fleas, ticks and other substances present in the environment. It is estimated that up to 8% of young children and 2% of adults have allergic reactions just to foods alone. Some allergic reactions (especially those to foods and insects) can be so severe as to be life threatening. Problems in animals tend to be less severe, but very common. For example, many dogs and cats have allergies to flea saliva proteins, grasses, and other common substances present in the environment.

Allergy is manifested by the release of histamines and other mediators of inflammation by mast cells which are triggered into action when IgE antibodies bound to their receptors on the mast cell surface are cross linked by antigen. Other than avoidance, and drugs (e.g. antihistamines, decongestants, and steroids) that only treat symptoms and can have unfortunate side effects and often only provide temporary relief, the only currently medically accepted treatment for allergies is immunotherapy. Immunotherapy involves the repeated injection of allergen extracts, over a period of years, to desensitize a patient to the allergen. Unfortunately, traditional immunotherapy is time consuming, usually involving years of treatment, and often fails to achieve its goal of desensitizing the patient to the allergen. Furthermore, it is not the recommended treatment for food allergies, such as peanut allergies, due to the risk of anaphylaxis.

Noon (Noon, *Lancet* 1911; 1:1572-73) first introduced allergen injection immunotherapy in 1911, a practice based primarily on empiricism with non-standardized extracts of variable quality. More recently the introduction of standardized extracts has made it possible to increase the

It is therefore an object of the present invention to provide a method for decreasing the allergenicity of allergens either by modifying the allergen itself or by producing a compound that would mask the epitope and thus prevent binding of IgE.

It is a further object of the present invention to provide allergens that elicit fewer IgE mediated responses.

It is still another object of the present invention to provide a method to make genetically engineered plants and animals that elicit less of an allergic response than the naturally occurring organisms.

Summary of the Invention

It has been determined that allergens, which are characterized by both humoral (IgG and IgE) and cellular (T cell) binding sites, can be made less allergenic by modifying the IgE binding sites. The IgE binding sites can be eliminated by masking the site with a compound that would prevent IgE binding or by altering as little as a single amino acid within the protein to eliminate IgE binding. The method allows the protein to be altered as minimally as possible, (i.e. only within the IgE-binding sites) while retaining the ability of the protein to activate T cells and, optionally, to bind IgG. Binding sites are identified using known techniques, such as by binding with antibodies in pooled sera obtained from individuals known to be immunoreactive with the allergen to be modified. Proteins that are modified to alter IgE binding are screened for binding with IgG and/or activation of T cells.

Peanut allergens (Ara h 1, Ara h 2, and Ara h 3) have been used in the examples to demonstrate alteration of IgE binding sites while retaining binding to IgG and activation of T cells. The critical amino acids within each of the IgE binding epitopes of the peanut protein that are important to immunoglobulin binding were determined. Substitution of even a single amino acid within each of the epitopes led to loss of IgE binding. Although the epitopes shared no common amino acid sequence motif, the hydrophobic residues located in the center of the epitope appeared to be most critical to IgE binding.

all of the Ara h 1 epitopes, whereas the open boxes represent the number of that type of residue which, when replaced, was found to result in the loss of IgE binding.

Figure 5 is a graph of the %IgE binding relative to wild type Ara h2 of modified Ara h 2 allergens.

Figure 6 shows the results of T-cell proliferation assays using the native and recombinant wild-type and modified Ara h 2 protein, compared to crude peanut as a control.

Detailed Description of the Invention

Definitions

The following definitions are used herein.

An antigen is a molecule that elicits production of antibody (a humoral response) or an antigen-specific reaction with T cells (a cellular response).

An allergen is a subset of antigens which elicits IgE production in addition to other isotypes of antibodies.

An allergic reaction is one that is IgE mediated with clinical symptoms primarily involving the cutaneous (uticaria, angiodema, pruritus), respiratory (wheezing, coughing, laryngeal edema, rhinorrhea, watery/itching eyes), gastrointestinal (vomiting, abdominal pain, diarrhea), and cardiovascular (if a systemic reaction occurs) systems.

An epitope is a binding site including an amino acid motif of between approximately six and fifteen amino acids which can be bound by either an immunoglobulin or recognized by a T cell receptor when presented by an antigen presenting cell in conjunction with the major histocompatibility complex (MHC). A linear epitope is one where the amino acids are recognized in the context of a simple linear sequence. A conformational epitope is one where the amino acids are recognized in the context of a particular three dimensional structure.

An immunodominant epitope is one which is bound by antibody in a large percentage of the sensitized population or where the titer of the

mediated through the interaction of IgE to specific proteins contained within the food. Examples of common food allergens include proteins from peanuts, milk, grains such as wheat and barley, soybeans, eggs, fish, crustaceans, and mollusks. These account for greater than 90% of the food allergies (Taylor, Food Techn. 39, 146-152 (1992). The IgE binding epitopes from the major allergens of cow milk (Ball, et al. (1994) *Clin. Exp. Allergy*, 24, 758-764), egg (Cooke, S.K. and Sampson, H.R. (1997) *J. Immunol.*, 159, 2026-2032), codfish (Aas, K., and Elsayed, S. (1975) *Dev. Biol. Stand.* 29, 90-98), hazel nut (Elsayed, et al. (1989) *Int. Arch. Allergy Appl. Immunol.* 89, 410-415), peanut (Burks et al., (1997) *Eur. J. Biochemistry*, 245:334-339; Stanley et al., (1997) *Archives of Biochemistry and Biophysics*, 342:244-253), soybean (Herein, et al. (1990) *Int. Arch. Allergy Appl. Immunol.* 92, 193-198) and shrimp (Shanty, et al. (1993) *J. Immunol.* 151, 5354-5363) have all been elucidated, as have others. Other allergens include proteins from insects such as flea, tick, mite, fire ant, cockroach, and bee as well as molds, dust, grasses, trees, weeds, and proteins from mammals including horses, dogs, cats, etc.

The majority of allergens discussed above elicit a reaction when ingested, inhaled, or injected. Allergens can also elicit a reaction based solely on contact with the skin. Latex is a well known example. Latex products are manufactured from a milky fluid derived from the rubber tree, *Hevea brasiliensis* and other processing chemicals. A number of the proteins in latex can cause a range of allergic reactions. Many products contain latex, such as medical supplies and personal protective equipment. Three types of reactions can occur in persons sensitive to latex: irritant contact dermatitis, and immediate systemic hypersensitivity. Additionally, the proteins responsible for the allergic reactions can fasten to the powder of latex gloves. This powder can be inhaled, causing exposure through the lungs. Proteins found in latex that interact with IgE antibodies were characterized by two-dimensional electrophoresis. Protein fractions of 56, 45, 30, 20, 14, and less than 6.5 kd were detected (Posch A. et al., (1997) J. *Allergy Clin. Immunol.* 99(3), 385-395). Acidic proteins in the 8-14 kd and 22 - 24 kd range that

mutagenesis by any of a number of techniques, to produce a modified allergen as described below, and thereby express modified allergens. It is also possible to react the allergen with a compound that achieves the same result as the selective mutation, by making the IgE binding sites inaccessible, but not preventing the modified allergen from activating T cells, and, in some embodiments, by not significantly altering or decreasing IgG binding.

Assays to assess an immunologic change after the administration of the modified allergen are known to those skilled in the art. Conventional assays include RAST (Sampson and Albergo, 1984), ELISAs (Burks, et al. 1986) immunoblotting (Burks, et al. 1988), and *in vivo* skin tests (Sampson and Albergo 1984). Objective clinical symptoms can be monitored before and after the administration of the modified allergen to determine any change in the clinical symptoms.

It may be of value to identify IgEs which interact with conformational rather than linear epitopes. Due to the complexity and heterogeneity of patient serum, it may be difficult to employ a standard immobilized allergen affinity-based approach to directly isolate these IgEs in quantities sufficient to permit their characterization. These problems can be avoided by isolating some or all of the IgEs which interact with conformational epitopes from a combinatorial IgE phage display library.

Steinberger et al. (Steinberger, P., Kraft D. and Valenta R. (1996) "Construction of a combinatorial IgE library from an allergic patient: Isolation and characterization of human IgE Fabs with specificity for the major Timothy Grass pollen antigen," Phl p. 5 *J. Biol. Chem.* 271, 10967-10972) prepared a combinatorial IgE phage display library from mRNA isolated from the peripheral blood mononuclear cells of a grass allergic patient. Allergen-specific IgEs were selected by panning filamentous phage expressing IgE Fabs on their surfaces against allergen immobilized on the wells of 96 well microtiter plates. The cDNAs were than isolated from allergen-binding phage and transformed into E coli for the production of large quantities of monoclonal, recombinant, allergen-specific IgE Fabs.

If native allergen or full length recombinant allergen is used in the

can be substituted for the original plant or animal, making immunotherapy unnecessary. Furthermore, it is possible that eating modified peanuts or cod fish, for example, could have either or both of two effects: (1) not imparting an allergic response on their own and (2) conferring protection from the unmodified source by acting as an immunotherapeutic agent for the unmodified source. Methods for engineering of plants and animals are well known and have been for a decade. For example, for plants see Day, *(1996) Crit. Rev. Food Sci. & Nut.* 36(S), 549-567, the teachings of which are incorporated herein. See also Fuchs and Astwood (1996) *Food Tech.* 83-88. Methods for making recombinant animals are also well established. See, for example, Colman, A" Production of therapeutic proteins in the milk of transgenic livestock" (1998) *Biochem. Soc. Symp.* 63, 141-147; Espanion and Niemann, (1996) *DTW Dtxch Tierarztl Wochenschr* 103(8-9), 320-328; and Colman, *Am. J. Clin. Nutr.* 63(4), 639S-6455S, the teachings of which are incorporated herein. One can also induce site specific changes using homologous recombination and/or triplex forming oligomers. See, for example, Rooney and Moore, (1995) *Proc. Natl. Acad. Sci. USA* 92, 2141-2149; Agrawal, et al., *BioWorld Today*, vol. 9, no. 41, p. 1"Chimeriplasty - Gene Surgery, Not Gene Therapy - Fixes Flawed Genomic Sequences" David N. Leff.

Production and Screening of Compounds blocking IgE Binding Sites

Once the IgE binding sites have been identified, it is also possible to block or limit binding to one or more of these sites by reacting the allergen with a compound that does not prevent the allergen from activating T cells, and in some embodiments does not significantly alter or decrease IgG binding capacity, resulting in a modified allergen similar in functionality to that produced by mutation. There are two principal ways to obtain compounds which block IgE binding sites: combinatorial libraries and combinatorial chemistry.

Identification of Compounds That Mask IgE Binding Sites through Application of Combinatorial Chemistry

In some cases it may be preferable to utilize non-peptide compounds

"Humanized" recombinant Fabs should bind to allergens if injected into a patient and thus prevent the binding of these allergens to native IgE. Since the Fabs cannot interact with the Fcε receptor, the binding of the IgE Fabs to allergen would not be expected to elicit mast cell degranulation. Allergen should be neutralized as it is by protective IgGs.

Anti-idiotype antibodies directed against the conformational epitope-specific Fabs should resemble the conformation epitopes themselves. Injection of these anti-idiotype antibodies should induce the production of anti-anti-idiotype IgGs which would recognize, bind to and inactivate the conformational epitopes. The method through which the anti-idiotype antibodies would be produced (i.e. animal immunization, "*in vitro*" immunization or recombinant phage display library) would have to be determined. Similarly, the possibility that the anti-idiotype antibodies (which resemble the conformational epitopes) would be recognized by patient IgEs and induce mast cell degranulation needs to be considered.

II. Diagnostic and Therapeutic Procedures Using Modified Allergens.

It is important to administer the modified allergen to an individual (human or animal) to decrease the clinical symptoms of allergic disease by using a method, dosage, and carrier which are effective. Allergen will typically be administered in an appropriate carrier, such as saline or a phosphate saline buffer. Allergen can be administered by injection subcutaneously, intramuscularly, or intraperitoneally (most humans would be treated by subcutaneous injection), by aerosol, inhaled powder, or by ingestion.

Therapy or desensitization with the modified allergens can be used in combination with other therapies, such as allergen-non-specific anti-IgE antibodies to deplete the patient of allergen-specific IgE antibodies (Boulet, et al. (1997) 155:1835-1840; Fahy, et al. (1997) *American J Respir. Crit. Care Med.* 155:1828-1834; Demoly, P. and Bousquet (1997) *J Am J Resp. Crit. Care Med.* 155:1825-1827), or by the pan specific anti-allergy therapy described in U. S. Serial No. 08/090,375 filed June 4, 1998, by M. Caplan

SEQ ID NOs. 1, 3, and 5, respectively. The amino acid sequences of Ara h 1, Ara h 2, and Ara h 3 are shown in SEQ ID NOs. 2, 4, and 6 respectively.

Example 1: Identification of linear IgE binding epitopes.

Due to the significance of the allergic reaction and the widening use of peanuts as protein extenders in processed foods, there is increasing interest in defining the allergenic proteins and exploring ways to decrease the risk to the peanut-sensitive individual. Various studies over the last several years have identified the major allergens in peanuts as belonging to different families of seed storage proteins (Burks, et al. (1997) *Eur. J. Biochem.* 245, 334-339; Stanley, et al. (1997) *Arch. Biochem. Biophys.* 342, 244-253). The major peanut allergens Ara h 1, Ara h 2, and Ara h 3 belong to the vicilin, conglutin and glycinin families of seed storage proteins, respectively. These allergens are abundant proteins found in peanuts and are recognized by serum IgE from greater than 95% of peanut sensitive individuals, indicating that they are the major allergens involved in the clinical etiology of this disease (Burks, et al. (1995) *J. Clinical Invest.*, 96, 1715-1721). The genes encoding Ara h 1 (SEQ ID NO. 1), Ara h 2 (SEQ ID NO. 3), and Ara h 3 (SEQ ID NO. 5) and the proteins encoded by these genes (SEQ ID NO. 2, 4, 6) have been isolated and characterized. The following studies were conducted to identify the IgE epitopes of these allergens recognized by a population of peanut hypersensitive patients and a means for modifying their affinity for IgE.

Experimental Procedures

Serum IgE. Serum from 15 patients with documented peanut hypersensitivity reactions (mean age, 25 yrs) was used to determine relative binding affinities between wild type and mutant Ara h 1 synthesized epitopes. The patients had either a positive double-blind, placebo-controlled, food challenge or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension; Burks, et al. (1988) *J. Pediatr.* 113, 447-451). At least 5 ml of venous blood was drawn from each patient, allowed to clot, and serum was collected. A serum pool from 12 to 15

bovine serum albumin overnight at 4°C. Primary antibody was detected with ^{125}I-labeled equine anti-human IgE (Kallestad, Chaska, MN).

Quantitation of IgE binding. Relative amounts of IgE binding to individual peptides were determined by a Bio-Rad (Hercules, CA) model GS-700 imaging laser densitometer and quantitated with Bio-Rad molecular analyst software. A background area was scanned and subtracted from the obtained values. Following quantitation, wild type intensities were normalized to a value of one and the mutants were calculated as percentages relative to the wild type.

Synthesis and purification of recombinant Ara h 2 protein. cDNA encoding Ara h 2 was placed in the pET-24b expression vector. The pET-24 expression vector places a 6 x histidine tag at the carboxyl end of the inserted protein. The histidine tag allows the recombinant protein to be purified by affinity purification on a nickel column (HisBind resin). Recombinant Ara h 2 was expressed and purified according to the instructions of the pET system manual. Briefly, expression of the recombinant Ara h 2 was induced in 200 ml cultures of strain BL21(DE3) E. coli with 1 mM IPTG at mid log phase. Cultures were allowed to continue for an additional 3 hours at 36°C. Cells were harvested by centrifugation at 2000 x g for 15 minutes and then lysed in denaturing binding buffer (6 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Lysates were cleared by centrifugation at 39,000 x g for 20 minutes followed by filtration though 0.45 micron filters. The cleared lysate was applied to a 10 ml column of HisBind resin, washed with imidazole wash buffer (20 mM imidazole, 6 M urea, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The recombinant Ara h 2 was then released from the column using elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The elution buffer was replaced with phosphate buffered saline by dialysis. The purification of recombinant Ara h 2 was followed by SDS PAGE and immunoblots. Peanut specific serum IgE was used as a primary antibody.

Skin prick tests. The ability of purified native and recombinant Ara h 2 to elicit the IgE mediated degranulation of mast cells was evaluated using

recognized by the majority of patients with peanut hypersensitivity, each set of epitopes identified for the peanut allergens were synthesized and then probed individually with serum IgE from 10 different patients. All of the patient sera tested recognized multiple epitopes.

Table 1 shows the amino acid sequence and position of each epitope within the Ara h 1 protein of all 23 IgE binding epitopes mapped to this molecule. Table 2 shows the amino acid sequence and position of each epitope within the Ara h 2 protein of all 10 IgE binding epitopes mapped to this molecule. Table 3 shows the amino acid sequence and position of each epitope within the Ara h 3 protein of all 4 IgE binding epitopes mapped to this molecule.

Four epitopes of the Ara h 1 allergen (peptides 1, 3, 4, 17 of Table 1), three epitopes of the Ara h 2 allergen (peptides 3, 6, 7 of Table 2), and 1 epitope of the Ara h 3 allergen (peptide 2 of Table 3) were immunodominant.

Table 1. Ara h I IgE Binding Epitopes

EPITOPE	AA SEQUENCE	POSITION
1	AKSSPYQKKT	25-34
2	QEPDDLKQKA	48-57
3	LEYDPRLVYD	65-74
4	GERTRGRQPG	89-98
5	PGDYDDDRRQ	97-106
6	PRREEGGRWG	107-116
7	REREEDWRQP	123-132
8	EDWRRPSHQQ	134-143
9	QPRKIRPEGR	143-152
10	TPGQFEDFFP	294-303
11	SYLQEFSRNT	311-320
12	FNAEFNEIRR	325-334
13	EQEERGQRRW	344-353
14	DITNPINLRE	393-402
15	NNFGKLFEVK	409-418
16	GTGNLELVAV	461-470
17	RRYTARLKEG	498-507
18	ELHLLGFGIN	525-534
19	HRIFLAGDKD	539-548
20	IDQIEKQAKD	551-560
21	KDLAFPGSGE	559-568
22	KESHFVSARP	578-587
23	PEKESPEKED	597-606

amino acids in length. The amino acids essential to IgE binding in each of the epitopes were determined by synthesizing duplicate peptides with single amino acid changes at each position. These peptides were then probed with pooled serum IgE from 15 patients with peanut hypersensitivity to determine if the changes affected peanut-specific IgE binding. For example, epitope 9 in Table 1 was synthesized with an alanine or methionine residue substituted for one of the amino acids and probed. The following amino acids were substituted (first letter is the one-letter amino acid code for the residue normally at the position, the residue number, followed by the amino acid that was substituted for this residue; the numbers indicate the position of each residue in the Ara h 1 protein, SEQ ID NO. 2): Q143A, P144A; R145A; K146A; I147A; R148A; P149A; E150A; G151A; R152A; Q143M; P144M; R145M; K146M; I147M; R148M; P149M; E150M; G151M; R152M. The immunoblot strip containing the wild-type and mutated peptides of epitope 9 showed that binding of pooled serum IgE to individual peptides was dramatically reduced when either alanine or methionine was substituted for each of the amino acids at positions 144, 145, and 147-150 of Ara h 1 shown in SEQ ID NO. 2. Changes at positions 144, 145, 147, and 148 of Ara h 1 shown in SEQ ID NO. 2 had the most dramatic effect when methionine was substituted for the wild-type amino acid, resulting in less than 1% of peanut specific IgE binding to these peptides. In contrast, the substitution of an alanine for arginine at position 152 of Ara h 1 shown in SEQ ID NO. 2 resulted in increased IgE binding. The remaining Ara h 1 epitopes, and the Ara h 2 and Ara h 3 epitopes, were tested in the same manner and the intensity of IgE binding to each spot was determined as a percentage of IgE binding to the wild-type peptide. Any amino acid substitution that resulted in less than 1% of IgE binding when compared to the wild type peptide was noted and is indicated in Tables 4-6. Table 4 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 1 epitopes. Table 5 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 2 epitopes. Table 6 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 3 epitopes.

This analysis indicated that each epitope could be mutated to a non-

Table 4: Amino Acids Critical to IgE Binding of Ara h 1

EPITOPE	AA SEQUENCE	POSITION
1	AKS**S**P**Y**Q**K**KT	25-34
2	QEP**DDL**KQKA	48-57
3	LE**Y**D**P**RL**VY**D	65-74
4	GE**R**TR**GRQ**PG	89-98
5	PGDYDD**D**RRQ	97-106
6	PRREE**G**GRWG	107-116
7	REREED**WRQ**P	123-132
8	EDW**RRP**SHQQ	134-143
9	Q**P**R**K**I**R**PEGR	143-152
10	T**P**GQ**F**ED**FF**P	294-303
11	S**YL**Q**EF**SRNT	311-320
12	**F**NAE**F**NEIRR	325-334
13	EQEER**G**QRRW	344-353
14	DIT**NP**I**N**L**R**E	393-402
15	NNFGK**L**F**F**EVK	409-418
17	**RRY**TARLKEG	498-507
18	EL**H**L**L**G**FG**IN	525-534
19	HRIFLAGD**K**D	539-548
20	IDQ**I**EKQ**A**K**D**	551-560
21	KDLA**FPG**SGE	559-568
22	KESHFV**S**ARP	578-587

Note. The Ara h 1 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 1 protein is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined residues. Epitopes 16 and 23 were not included in this study because they were recognized by a single patient who was no longer available to the study. All of these sequences can be found in SEQ ID NO 2.

As shown by Figure 4, hydrophobic amino acids are more critical to IgE binding. The type of each amino acid within the Ara h 1 epitopes was assessed relative to its importance to IgE binding. The closed boxes represent the total number of a particular type of amino acid residue found in all of the Ara h 1 epitopes, whereas the open boxes represent the number of that type of residue which, when replaced, was found to result in the loss of IgE binding.

Table 6. Amino Acids Critical to IgE-Binding of Ara h 3.

EPITOPE	AA SEQUENCE	POSITION
1	IETWN**PN**NQEFECAG	33-47
2	GNI**F**SG**F**TPE**FL**EQA	240-254
3	VTVRGG**L**R**IL**S**P**DRK	279-293
4	DEDEY**EYDE**E**DR**RRG	303-317

Note. The Ara h 3 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 3 protein is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined All of these sequences can be found in SEQ ID NO 6.

IgE-binding peptides 1-4 were synthesized and probed individually with serum igE from 20 peanut-hypersensitive patients. The percentage of individual peanut-hypersensitive patients recognizing epitopes 1-4 ranges from 5% to 100%. The IgE-binding sequence and its corresponding position in the primary sequence of Ara h 3 is also shown in Table 7.

Table 7. Percentage of recognition of Ara 3 Peptides

Sequence	Position	Precentage
EQEFLRYQQQ	183-192	5% (1/2)
FTPEFLEQAF	246-255	25% (5/20)
EYEYDEEDRR	300-309	35% (7/20)
LYRNALFVAH	379-388	100% (20/20)

Example 3: A Modified Ara h 2 Protein Binds less IgE But Similar Amounts of IgG.

In order to determine the effect of changes to multiple epitopes within the context of the intact allergen, four epitopes (including the three immunodominant epitopes) of the Ara h 2 allergen were mutagenized and the protein produced recombinantly. The amino acids at position 20, 31, 60, and 67 of the Ara h 2 protein (shown in SEQ ID NO. 4) were changed to alanine by mutagenizing the gene encoding this protein by standard techniques. These residues are located in epitopes 1, 3, 6, and 7 and represent amino acids critical to IgE binding that were determined in Example 2. The modified and wild-type versions of this protein were produced and

determine the T cell epitopes of Ara h 2. Peanut specific T cell lines were established from the peripheral blood of 12 atopic patients and 4 nonatopic controls. All of the cell lines were shown to consist of predominantly CD4+ T cells. The proliferation of the T cells in response to the 29 individual peptides was measured. Four immunodominant T cell epitopes were identified for Ara h 2, epitope 1 (AA 18-28), epitope 2 (AA 45-55), epitope 3 (AA 95-108), and epitope 4 (134-144). Epitopes 1, 2, and 4 have overlapping sequences with Ara h 2 B cell epitopes whereas epitope 3 does not overlap IgE binding epitopes, providing the possibility for the development of a non-anaphylactic, T cell directed, immunotherapeutic peptide.

This process was repeated with T cells isolated from 17 peanut allergic individuals and 5 non-peanut allergic individuals, placed in to 96 well plates at 4 x 10^4 cells/well and treated in triplicates with media or Ara h 2 peptides (10 micrograms/ml). The cells were allowed to proliferate for 6 days and then incubated with ^{3}H-theymidine (1 microCi/well) at 37 C for 6-8 hrs and then harvested onto glass fiber filters. T cell proliferation was estimated by quantitating the amount of ^{3}H-thymidine incorporation into the DNA of proliferation cells. ^{3}H-thymidine inocrporation is reported as stimulation (SI) above media treated control cells. Graphs of the proliferation of T cells (x-axis) from each individual plotted versus the 29 overlapping peptides (y-axis) spanning the entire Ara h 2 protein from the amino to carboxyl terminus (peptide 932) were prepared.

T cells were stained with FITC-labeled anti-CD4 and FITC-labeled anti-CD8 antibodies in order to determine the phenotype of the peanut specific T-cell lines established. FACS analysis was used to determine the precent of CD4+ and CD8+ cells in the peanut specific T-cell lines utilized in Ara h 2 epitope mapping and ploted versus the initials of the individual patients used to establish these cell lines. The supernatant was collected from T-cells stimulated with immunodominant peptides and an ELISA assay was utilized to measure IL-4 concentrations in the media. IL-4 concentration was plotted versus the 29 overlapping peptides spanning the entire Ara h2 protein from amino to carboxyl terminus.

that developed was measured. The wheal and flare produced by the wild-type Ara h 2 protein (8 mm X 7 mm) was approximately twice as large as that produced by the modified Ara h 2 protein (4 mm X 3mm). A control subject (no peanut hypersensitivity) tested with the same proteins had no visible wheal and flare but, as expected, gave positive results when challenged with histamine. In addition, the test subject gave no positive results when tested with PBS alone. These results indicate that an allergen with only 40% of its IgE binding epitopes modified (4/10) can give measurable reduction in reactivity in an *in vivo* test of a peanut sensitive patient.

These same techniques can be used with the other known peanut allergens, Ara h 1 (SEQ ID NO 1 and 2), Ara h 3 (SEQ ID NO. 5 and 6), or any other allergen.

Example 8: IgE binding sites may be blocked by or formed in part by carbohydrate structures.

Studies demonstrated that the Ara h 1 trimer, which is stable at high salt concentration, is unstable at an acidic pH (2.1) that is found in the human stomach. The allergen was digested with pepsin, trypsin, and chymotrypsin.

Purified Ara h 1 (9.5 microM) was subjected to digestion with trypsin (0.01 microM) at 37C for varying lengths of time up to three hours. Samples were withdrawn at various times and prepared for analysis.

Portions were resistant to digestion. These peptides contain IgE binding epitopes, as demonstrated by immunoblot analysis using a pool of serum IgE from peanut sensitive patients. Immunoblot analysis with an antibody that recognizes a unique carbohydrate structure that includes a beta-1,2-linked xylose attached to the beta-linked mannose of the core oligosaccharide chain showed protease resistant fragments in all samples for up to three hours after addition of the enzyme, many of which were glycosylated. Most peptides of the protease-resistant Ara h 1 peptides contain a beta-1,2-linked xylose attached to the beta-linnked mannose of the core oligosaccharide chain.

9. The method of claim 1 wherein the modified allergen is screened for initiation of a T cell helper 1 response.

10. The method of claim 1 wherein the modified allergen is made in a recombinant host selected from the group consisting of plants, animals, bacteria, yeast, fungi, and insect cells.

11. The method of claim 1 wherein the modified allergen is made in cells using site specific mutation.

12. The method of claim 1 wherein the modified allergen is made from a peanut allergen selected from the group consisting of Ara h 1, Ara h 2, and Ara h 3.

13. The method of claim 1 wherein the modified allergen is based on a protein obtained from a source selected from the group consisting of legumes, milks, grains, eggs, fish, crustaceans, mollusks, insects, molds, dust, grasses, trees, weeds, mammals, birds, and natural latexes.

14. A modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change or having at least one amino acid bound by a compound so that the site no longer binds IgE, wherein the modified allergen activates T cells.

15. The modified allergen of claim 14 wherein the modified allergen binds IgG.

16. The modified allergen of claim 14 made by the process of

(a) identifying one or more IgE binding sites in an allergen;

(b) mutating at least one amino acid in an IgE binding site;

(c) screening for IgE binding to the mutated allergen and activation of T cells by the mutated allergen; and

(d) selecting the modified allergens with decreased binding to IgE which activate T cells.

17. The modified allergen of claim 14 wherein the modified allergen is mutated in the center of one or more of the IgE binding sites.

30. A nucleotide molecule encoding a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells.

31. The molecule of claim 30 in a vector for expression in a recombinant host.

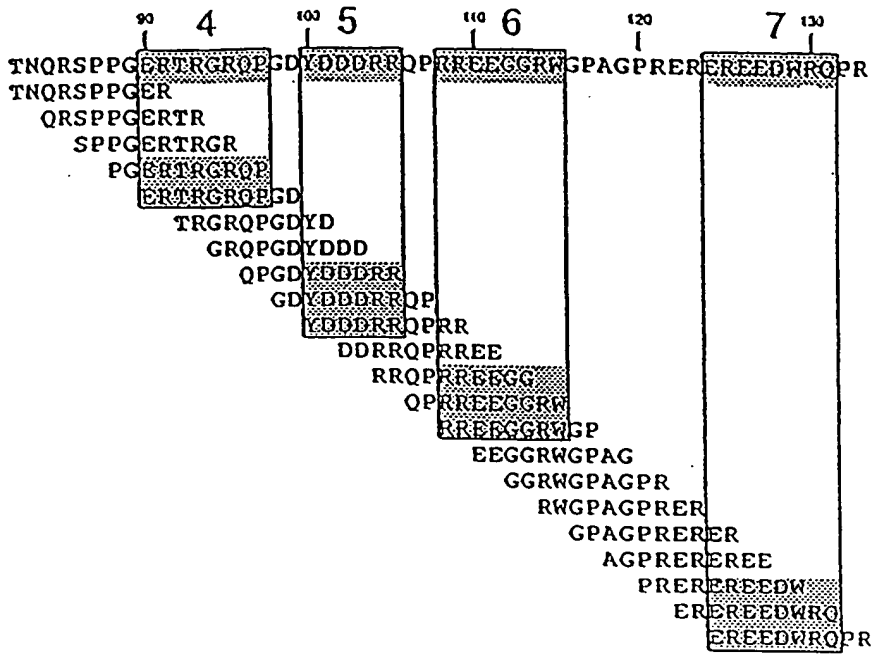
32. A nucleotide molecule for causing a site specific mutation in a gene encoding a protein which yields a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells.

33. A transgenic plant expressing a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells.

34. A transgenic animal expressing a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells.

35. A compound selectively binding to at least one amino acid in an IgE binding site of an allergen, wherein the site no longer binds IgE, but wherein the allergen is able to activate T cells, wherein the compound is obtained using a combinatorial library or combinatorial chemistry and screening for reaction with the allergen to produce bound allergen, followed by testing of the bound allergen for binding to IgE and activation of T cells.

36. A method to treat an individual to reduce the clinical response to an allergen comprising administering to the individual a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change or having at least one amino acid bound by a compound so that the site no longer binds IgE, wherein the modified allergen activates T cells, in an amount and for a time sufficient to reduce the allergic reaction to the unmodified allergen.


90
4
100
5
110
6
120
7
130
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QRSPPGERTR
SPPGERTRGR
PGERTRGRQP
ERTRGRQPGD
TRGRQPGDYD
GRQPGDYDDD
QPGDYDDDRR
GDYDDDRRQP
YDDDRRQPRR
DDRRQPRREE
RRQPRREEGG
QPRREEGGRW
RREEGGRWGP
EEGGRWGPAG
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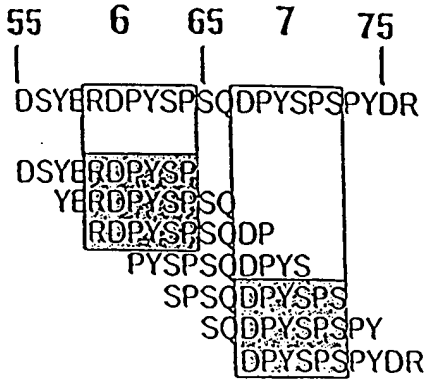
PRERERREDW
ERERREEDWRQ
ERREEDWRQPR

FIG. 1


55
6
65
7
75
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SPSQDPYSPS
SQDPYSPSPY
DPYSPSPYDR

FIG. 2

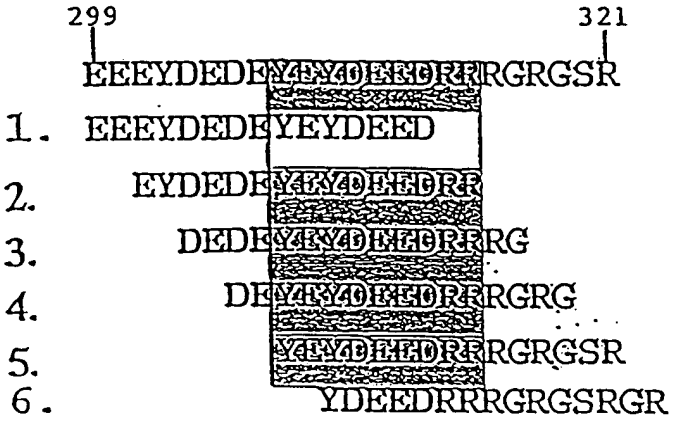
299
321
EEEYDEDEYEYDEEDRRRGRGSR
1. EEEYDEDEYEYDEED
2. EYDEDEYEYDEEDRR
3. DEDEYEYDEEDRRRG
4. DEYEYDEEDRRRGRG
5. YEYDEEDRRRGRGSR
6. YDEEDRRRGRGSRGR

FIG. 3

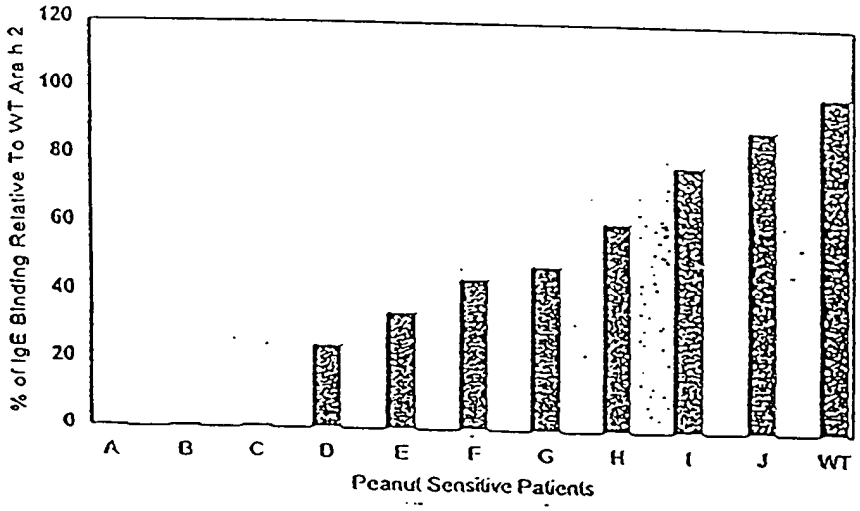
% of IgE Binding Relative To WT Ara h 2
120
100
80
60
40
20
0
A
B
C
D
E
F
G
H
I
J
WT
Peanut Sensitive Patients

FIG. 5

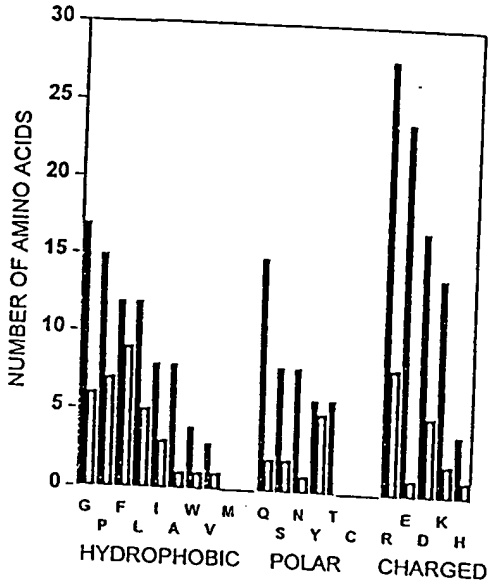

FIGURE 4

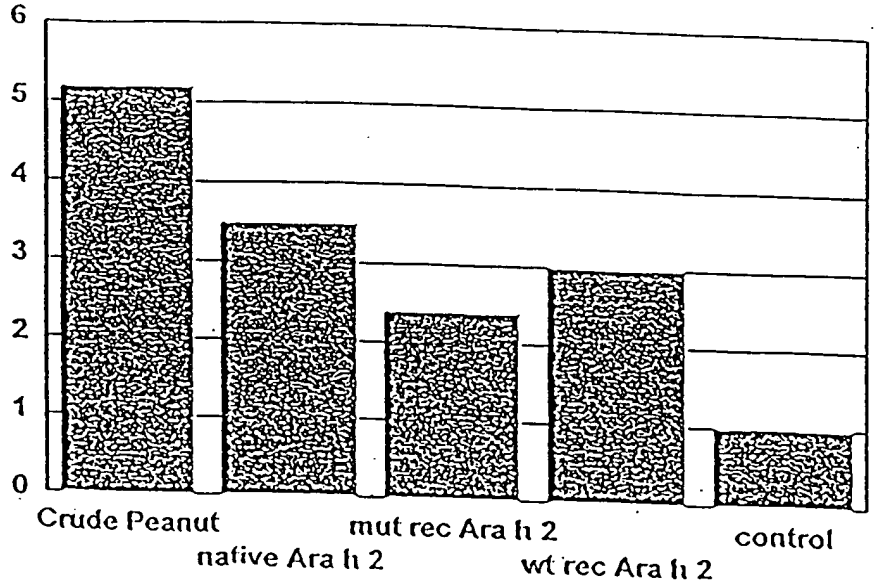

FIG. 6